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Cryopreservation of Fish Sperm

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Summary

This chapter provides detailed descriptions of the protocol used for fish sperm cryopreservation. The principles described can be applied to any species, but details are also given for individual fish species. Successful cryopreservation of fish spermatozoa depends on a range of factors including the collection of high quality sperm, equilibration conditions, choice of cryoprotectant medium, cooling/thawing regimes, and conditions for fertilization. Even though some general rules can be applied to any fish species, optimization of the protocol is needed for each individual species. Furthermore, because sperm derived from marine and freshwater fish differs markedly, the described protocol is mainly focused on the cryopreservation of sperm for freshwater fish because this poses the greater technical challenge.

Key Words: Fish sperm cryopreservation; freshwater species; carps; salmonids; sturgeon.

1. Introduction

The development of routine use of "artificial" fertilization in aquaculture has raised the requirement for storage of reproductive materials. The short period of time for which fish gametes remain in good condition after collection became a significant obstacle to hybridization between species of fish inhabiting different geographical locations, or having different spawning times. The asynchronous maturation of breeders invariably causes problems in aquaculture and has stimulated research to develop a method suitable for the prolonged storage of fish sperm. Several different approaches were initially tested including storage of fish sperm in medium saturated with different gases ***(1)***, preservation of sperm at temperatures above zero ***(2)***, as well as in the frozen state ***(3)*** and drying ***(4)***. However, to date, low-temperature preservation has proven to be the most effective approach, with the first successful cryopreservation of fish sperm reported by Blaxter in 1953 ***(3)***. The method has subsequently been applied

From: *Methods in Molecular Biology, vol. 368: Cryopreservation and Freeze-Drying Protocols, Second Edition*
Edited by: J. G. Day and G. N. Stacey © Humana Press Inc., Totowa, NJ

widely and has become not only a routine tool in aquaculture for fish hybridization and selective breeding, but also an important tool in programs on biodiversity and the preservation of endangered species. Gamete banks of rare or almost extinct species are currently being created ***(5)*** with the objective of protecting endangered species. The technique has also found applications in research programs for maintaining laboratory animals and, in recent years, sperm of more than 200 species of fish have been successfully cryopreserved ***(6)***. However, despite the extensive number of studies that have been undertaken there is still ambiguity in the data reported in the literature, primarily because of poor standardization of methodology and data analysis.

Male reproductive cells derived from fish are significantly different than mammals', not least because the gametes of the vast majority of fish are fertilized externally, their spermatozoa need activation (fish spermatozoa remain immotile until they are expelled into water), and have a relatively short duration of motility. Fish inhabit almost all surface waters on Earth ranging from freshwater to hypersaline lakes and cold Arctic water to hot waters of the California desert ***(7)***. Because fish have evolved to live in these diverse environments, there are substantial differences in fish morphofunctional characteristics. Fish have had to develop adaptation mechanisms to survive in these diverse environmental conditions, and as a result spermatozoa from fish species demonstrate significant differences in their reactions to cryopreservation protocols. For example, there is a striking difference in post-thaw survival of reproductive cells of marine and freshwater species. Sperm of marine species were successfully cryopreserved ***(3)*** soon after the discovery of the first cryoprotectant, whereas the cryopreservation of freshwater fish gametes was more challenging and took longer to achieve ***(8–10)***.

Although there are many protocols available for low-temperature storage of sperm of freshwater fish ***(11,12)*** there is much work still to be done to improve this technology. In general, approx 40–90% of spermatozoa from freshwater species are usually damaged after cryopreservation, whereas only 10–20% of spermatozoa are damaged in marine species ***(11)***. The difference between species is presumably a result of evolution in their cellular properties developed under the pressure of their niche environment. In the author's experience, post-thaw survival of fish sperm is strongly predetermined by their sensitivity to osmotic changes in extracellular media. Freshwater species inhabit an environment of 0–50 mOsm, whereas marine fish live in a range of 600–1000 mOsm. Consequently, the osmolality of the seminal plasma differs between species, for example, Siberian sturgeon (*Acipenser baeri*) having semen of 20 to 60 mOsm, whereas most of freshwater or seawater teleost has semen osmolarity between 230 and 320 mOsm ***(13)***. The activation of freshwater sperm is because of a

decrease in extracellular osmolarity, and sperm originating from saltwater species is activated by an increase in external osmolarity ***(14)***. The sperm of marine species is not damaged by an environment with an increased osmolarity and it can easily tolerate the increased osmolarity it experiences during cryoprotectant treatment and cryopreservation. Sperm from freshwater fish are not naturally accustomed to an increased osmolarity, and thus are subjected to significant stress during equilibration with cryoprotectants and subsequent cryopreservation.

High variability in cryoresistance is reported not only between species of fish, but also between individual males ***(15)***. In this chapter, it is our intention to summarize the factors that, in our opinion, are important in achieving a successful protocol. It is unfortunately impossible to offer a single universal protocol for such a diverse kingdom as fish. Instead, examples of successful protocols used for cryopreservation of the most widely studied species of freshwater fish are given. To develop reliable protocols of cryopreservation for fish spermatozoa, individual fish and species-specific properties have to be taken into consideration. Important factors that can undermine the final quality are outlined and these should be taken into consideration when a new protocol is being developed for a previously unstudied species.

2. Materials

1. Gamete materials: sperm and a suspension of sperm cells prepared from testes. Fresh eggs collected immediately before fertilization.
2. Solutions.
   a. Extenders (solutions without cryoprotectant): the choice of appropriate extender depends on the species to be investigated (*see* **Note 1**).
   b. Freshly prepared cryoprotectant solutions. The most commonly used cryoprotectants for fish sperm cryopreservation are dimethyl sulfoxide (DMSO), ethylene glycol, methanol, ethanol, glycerol, and dimethylacetamide. The concentration usually varies between 5 and 12% (w/v). Sucrose, polyethyleneglycol, albumin, and hen egg yolk have also been used (*see* **Note 1**).
   c. Activating medium for fertilization: 25 m*M* Tris and 150 m*M* NaCl medium can be used for sturgeons and carp ***(12)***; 60 m*M* $NaHCO_3$, 20 m*M* glycine, 5 m*M* theophylline, 50 m*M* Tris-HCl (pH 9.0) is used for Salmonid fishes ***(16)***.
3. Containers for milt collection: vials, flasks, tissue culture flasks. The type and size of container depends on species and volume of collected sperm (*see* **Note 2**).
4. Container with ice.
5. Cryocontainers: cryovials and straws.
6. Cryostorage equipment.
7. Water bath.
8. Refrigeration systems:

   a. Programmable variable-rate cooler.
   b. Wide-necked Dewar (10–15 L) with liquid nitrogen. The neck diameter of 10–15 cm.
   c. Refrigerated alcohol cold baths (for –20 and –40°C).
   d. Polystyrene box with dry-ice.
9. Thermocouple and device for monitoring temperature changes.
10. Storage system: crystorage containers with appropriate storage racks and inventory system suitable for holding cryovials.
11. Microscope with a computer-assisted semen analysis system.
12. Petri dishes for fertilization.
13. Low temperature-resistant markers for glass.
14. Hemocytometer.

3. Methods

1. Select the appropriate breeders and prepare them, if necessary, for spawning (*see* **Note 3**).
2. Stimulation of maturation. Unless naturally spawning fish are used, artificial stimulation is required. In order to obtain good quality sperm, keep fish under optimal conditions and avoid fluctuations and/or changes of temperature after stimulation and during spermiation. Inject chorionic gonadotropin or solution of the pituitary gland. The dose depends on species, fish weight, degree of maturation, and the quality and type of injected hormones. For examples of dosage refer to **Table 1** (*see* **Note 4**). The injections are made intramuscularly in the cranial part of the body, or under the one of pectoral fins (*see* **Note 5**).
3. Collection of milt.
   a. Collect sperm from mature males into clean and dry flasks applying gentle abdominal massage. For small males it can be useful to sedate the fish (with 25 mg/L tricaine methanesulfate [MS-222]) and use a capillary tube. The collection of sperm from large fish such as sturgeon is performed on a specially designed table. The table has two surfaces under a 90° angle and a 10° slope with soft cover. A tube with flowing water is inserted into the fish's mouth, while a transparent polyethylene tube is placed into its genital orifice. This method of sperm collection helps to prevent sperm contamination and traumatization of the fish (*see* **Note 6**).
   b. Collection of sperm by sacrificing the fish. This approach is usually only necessary in the case of small aquarium fish or in some species where the collection of liquid sperm is not possible without extraction of testes (for example, Weather Loach *Misgurnus fossilis*). Anesthetize fish by placing them into a solution of 100 mg/L tricaine methanesulfate or 25–50 mg/L benzocaine. Extract testes from abdominal cavity and transfer them to a dry, clean dish.
4. Maintain the milt samples obtained at 4–5°C, or on ice in well-aerated flasks in a thin layer of sperm sample. It is preferably not to store sperm samples after collection, one should proceed immediately with cryopreservation (*see* **Notes 7** and **8**).

Table 1
Examples of Hormone Doses Used for Stimulation of Fish Spermiation

Species	Injected material	Dose
Sturgeon (*Acipenser baeri*)	Powder of sturgeon pituitary gland dissolved in water	2 mg/kg
Carp (*Cyprinus Carpio*)	Powder of carp pituitary gland dissolved in water	4 mg/kg
Loach (*Misgurnus fossilis*)	Chorionic hormone	100 U/fish

5. Inspection of cell quality under the microscope (*see* **Note 9**). Activate cell movement by addition of the appropriate activating medium. Fresh sperm can be activated by water from the aquarium tank after dilution not less than in 40 times followed by good, rapid mixing. Sperm with low motility rate and with low cell density should not be used for cryopreservation. For activation after cryopreservation, *see* **step 15** (*see* **Note 10**). If necessary, cell density can be established with the help of a hematocytometer.
6. Preparation of milt suspension if **step 2** was used. Prepare sperm suspension from extracted testes. Care must be taken to avoid milt contact with water, urine, blood, and feces to prevent activation and contamination of sperm. Testes are cut into small pieces and homogenized with the extender.
7. Dilute sperm with appropriate cryoprotectant (*see* **Notes 11** and **12**). Establish the appropriate ratio of dilution for each species (*see* **Note 13**). Cryoprotectant solutions that causes more than a 50% decline in sperm motility rate must not be used. Cryoprotectants should be added very slowly and gradually (*see* **Note 14**) with a constant mixing of sperm with the added solution. The concentration of cryoprotectant usually varies in the range between 5 and 12% (v/v).
8. Equilibration. Immediately after sperm collection, it is cooled down to 5°C and diluted with isothermal cryoprotectant medium. The duration of exposure to the cryoprotectant solution at 5°C is generally in the range of 20 to 60 min. However, for some species equilibration might not be necessary (*see* **Note 15**).
9. Load samples in 1–2 mL vials. The volume of the cryopreserved sample should not usually exceed 2 mL (*see* **Note 2**). The smaller the volume that is used, the easier it is to achieve the desired freezing regime within the sample. Leave at least one-fourth of ampoule volume free of sample (*see* **Note 16**).
10. Freezing.
    a. Freezing in vapor-phase liquid nitrogen (*see* **Note 17**). Place vials or straws above the liquid nitrogen horizontally on the rack at a predetermined height. The height of sample placement and the time of exposure at this height depend on sample volume, type of container, type of the Dewar, and temperature at that height. For example, samples of sturgeon sperm in 12% (v/v) DMSO placed in vials in 0.7- or 1.5-mL volumes are kept at 20-cm above the surface

of liquid nitrogen for 20 min ***(17)***. During this time sperm is cooled down to –20°C. After that samples are moved at 2-cm above the liquid nitrogen level for 5 min followed by plunging into liquid nitrogen.

Sperm of salmonid fish is cryopreserved in straws with volumes of 0.5 or 1.2 mL ***(16)***. Straws containing 0.5 and 1.2 mL are frozen at 1.5- and 2.5-cm above the level of liquid nitrogen (LN) respectively, with a holding time of 10 min ***(16)***.

b. Freezing in alcohol baths. Similar results can be obtained by freezing sperm in cold baths that are capable of maintaining a set temperature. Prepare two cold baths precooled to –20 and –40°C. Vials (0.7 or 1.5 mL) are placed in the bath at –20°C for 2.5 min and then are transferred to a bath at –40°C for 2.5 min. The samples are then plunged into liquid nitrogen.

c. Freezing in dry-ice. Place small aliquots (100–250 μL) of sperm diluted with cryoprotectant directly onto dry ice and allow them to cool for 4–5 min. Then transfer to cryovials and store in liquid nitrogen.

d. Controlled-rate cooling. The following protocol can be applied for sperm of different species. The first step from 0 to –15°C involves cooling at 1–5°C/min (1–2°C/min for Carp); second step from –15 to –70°C cooling at 15–20°C/min, and the third step plunging into liquid nitrogen (*see* **Note 18**).

11. Store in liquid nitrogen (*see* **Note 19**).
12. Thawing. Transfer the vials or straws from liquid nitrogen directly into a water bath at 40°C (*see* **Note 20**). Rotate the vials while they are immersed in a water bath until the appearance of the liquid phase. Then shake the vials in the air until no ice remains.

    For samples frozen as pellets, add them directly to eggs along with the activator, the temperature of which would be the same as river (or tank) water from which the fish originated. In the case of carp, the thawed sperm samples could be stored on ice and the fertility rate could be improved after some additional treatment (*see* **Note 21**).
13. "Rehabilitation" of sperm. Aerate sperm from carp for 5–15 min after thawing before fertilization (*see* **Note 21**).
14. Fertilization. Ensure that the eggs are ready for fertilization. Place sperm samples directly into a container with eggs together with activating medium. Use 42 m*M* $NaHCO_3$ or NaCl as an activating media (*see* **Note 10**). If available use a goose feather for mixing eggs if a large quantity is to be used.
15. Assessment of sperm quality after cryopreservation. Use microscope assessment as outlined in **step 5**, plus fertilization and hatching rate for assessment of sperm quality.
16. Keep detailed comprehensive records of the cryopreserved materials and protocol.

4. Notes

1. The choice of extender depends on the species from which the sperm is obtained (*see* **Table 2**).
2. Good results are usually obtained when fish sperm is frozen in granules, straws, and/or plastic vials of 0.05–2 mL volume. Larger volumes create difficulties in achieving uniformed freezing of the sample. Containers larger than 5 mL are not normally recommended for the cryopreservation of fish sperm.

Table 2
Example of Cryoprotectant Media for Some Species

Fish species	Medium ingredients	Quantity
Carp (*Cuprinus Carpio*) ***(18)***	NaCl	42 mg
	KCl	6 mg
	$CaCl_2 \cdot 6H_2O$	18 mg
	$MgSO_4 \cdot 7H_2O$	62 mg
	$NaHCO_3$	280 mg
	Sucrose	137 mg
	D-Mannitol	1.5 g
	Tris-oxymethyl-aminomethane basis	1.697 g
	Glutathione red	56 mg
	Polyvinyl alcohol	5 mg
	Hen egg yolk	12.0 mL
	HCl	Adjust pH 8.1
	H_2O	up to 100 mL
	Ethylene glycol	19.6 mL
Sturgeon fish ***(17)***	Tris-HCl buffer	0.05 *M*
	Egg yolk	20%
	DMSO	25% (after dilution 1:1 final concentration 12.5%)
Salmonid fish ***(16)***	NaCl	600 mg
	KCl	315 mg
	$CaCl_2 \cdot 2H_2O$	15 mg
	$MgSO_4 \cdot 7H_2O$	20 mg
	HEPES	470 mg
	H_2O	Up to 100 mL
	Methanol	10 mL
	Bovine serum albumin	1.5 g
	Sucrose	0.5 g
	Hen egg yolk	7 mL

3. The success of cryopreservation strongly depends on the initial quality of the sperm. Higher quality material can be obtained from matured breeders in the middle of a breeding season ***(19,20)***. The quality of sperm drops substantially by the end of the spawning period in trout, herring, and others species ***(21)***. The recommended motility rate of sperm that can be used for cryopreservation should ideally not be lower than 80%. For example, the sperm of sturgeon with a motility rate lower than 40% should not be used for cryopreservation. Sturgeon sperm with low motility and cell concentration is not recommended to be used for fertilization, even without cryopreservation ***(2)***. Better quality sperm can

usually be obtained in the morning, when natural spawning takes place for the majority of species.

4. A variety of hormones and doses may be employed (*see* **Table 1**).
5. The injecting is performed slowly and gentle rubbing to the site of injection is applied afterward to avoid injected hormone leaking out because of muscle constriction. It is optimal to inject the fish in the morning. After injection place the fish back into water at their optimal light and temperature regime. Spermiation normally occurs 12–43 h after injection, depending on species and spawning temperature.
6. It is of paramount importance to avoid sperm contamination with water, urine, and feces during fish stripping. As it was demonstrated in salmon (*Salmo salar*) ***(22)***, the collection of sperm by abdominal pressure causes urine contamination that can dilute milt by as much as 80% (v/v). This causes substantial variability of sperm osmolarity, a decrease of K^+ concentration, and motility. The contamination of sperm with up to 25% (v/v) urine causes the one-third decrease in motility after cryopreservation in comparison with uncontaminated samples. Other authors ***(23)*** also reported the decrease of survival in cryopreserved sperm of Atlantic salmon (*Salmo salar*) after mixing it with different amounts of urine. Therefore, it is recommended ***(24)*** that one wipes the abdominal part of a male thoroughly before collecting a sperm sample to clear any water and to expel urine by gentle abdominal pressure. It is more reliable to use catheters for sperm collection, this minimizes contamination. Feces in sperm also affects the motility rate. Clean, uncontaminated, sperm samples from the Walleye (*Stizostedion vitreum*) could be stored almost six times longer (11.7 d) than sperm contaminated with feces (2 d) ***(25)***. Contamination of sperm with blood also decreases the duration of possible storage, as well as the motility rate. Bacterial contamination has also been demonstrated, in our laboratories, to have a negative impact on the functional stability of cells. Antibiotic treatment helps significantly to extend the duration of hypothermal storage in Stellate sturgeon (*Acipenser stellatus*) ***(26)***. The risk of sperm contamination can be one of the reasons why cryopreservation is recommended straight after sample collection without any preliminary storage.
7. The container for holding sperm samples can also affect the quality of sperm. Tall and narrow containers must be avoided to minimize the possibility of hypoxic conditions developing in the holding container. The conditions for short-term storage/holding of sperm samples need to provide the best possible conditions for appropriate access of oxygen, and the use of wide-neck containers is optimal. Depending on sperm concentration the depth of sperm sample should be in a range of 5 to 15 mm. For example, sturgeon sperm placed into a fridge in closed vials, without access of oxygen, loses motility in 3 h. Samples kept in refrigerators in open humidifying containers, with a depth of less than 15 mm, preserve the sperm motility for as long as several days ***(26)***. Experiments with carp sperm also indicates that decrease of storage temperature and thickness of samples increase sperm survival (Kopeika, unpublished data). The layer of air above the sperm sample should be 7–10 times thicker than the layer of sperm. These conditions

are especially important when sperm is not going to be used immediately for cryopreservation.

8. In order to decrease the energy lost of spermatozoa during maintenance, the temperature of temporary storage needs to be approx 0–5°C. If ice is used for temporary storage, direct contact between ice and sample needs to be avoided. Sperm of sturgeon (*Acipenser guldenstadti*) stored at 1–4°C maintained fertilizing ability for 5–6 d ***(27)***, whereas when stored in ice fertilizing ability was maintained for up to 8 d ***(2)***. Prolonged storage at 1–4°C was also shown to be effective for many other species of fish ***(28)***. However, storage at temperatures of 1–4°C is not adequate for the long-term preservation of sperm.
9. Motility assessment. The percentage of motile sperm can be assessed by light microscopy. However, the operator requires experience to assess the motility rate accurately. Therefore, where available, a computer-assisted semen analysis system should be used wherever possible to standardize measurement of motility. This system not only allows the accurate estimation of sperm motility, but also a range of other valuable measurements for the assessment of sperm quality. Adequate assessment of sperm quality also depends on their appropriate dilution. It has been demonstrated for sperm of trout, carp ***(29)***, and Siberian sturgeon ***(30)*** that after low levels of dilution not all sperm were activated simultaneously and the process of activation can last from 15 s to 3 min after dilution. It is advised that in some cases two-step dilution should be used; first dilute sperm with nonactivating medium and then dilute sperm with activating medium. The degree of dilution needs be optimized for each species. As a general approach we recommend that 5 µL of sperm should be activated by 2 mL H_2O for "native" sperm, or activating medium for freeze–thawed sperm. If larger volumes of sperm need to be assessed, then two-step dilution is preferable ***(30)***. First, dilution with a 1:20 ratio in nonactivation solution of 400 m*M* sucrose, 20 m*M* Tris (pH 8.0) (sperm remains immotile); second, activation of sperm using 30 m*M* Tris (pH 8.0).
10. By the time sperm is thawed and ready to be used for fertilization they have gone through a range of stresses. Further treatments, such as activation of motility in pure water, can also affect functional activity of weak sperm cells post-thaw. Therefore, care has to be taken during handling of sperm after thawing and pure water should not be used as an activator for cryopreserved-thawed sperm during fertilization. Better activation will be attained in activation media that have higher osmolarity than pure water. However, the increase in osmolarity in the activating medium has to be within the range that is safe for the eggs. There are different media used for sperm activation (40 m*M* NaHCO3, or 60 m*M* NaCl + 50 m*M* Tris-HCl ***(12)***, Tris/NaCl/$CaCl_2$ 10/20/2 mmol/L, pH 8.5) ***(31)***. There is also some evidence that the addition of certain chemicals during, or immediately after, fertilization ***(32)*** with cryopreserved fish sperm can enhance the hatching rate.
11. Choice of extender. The osmolarity of the extender solution appears to be one of the most important factors in preparation of an appropriate extender. Because large interindividual differences have been demonstrated in fish semen osmotic responses, in order to maximize the outcome of cryopreservation the osmolarity

of extender should be adjusted on the individual bases if possible. Increased concentration of buffer or K^+ may be used to inhibit sperm motility in extenders for some species such as sturgeon.

12. Both permeable (glycerol, DMSO, ethylene glycol, dimethylacetamide, methanol) and nonpermeable (sucrose) cryoprotectants may be used in protocols to cryopreserve fish sperm. The absence of an ideal cryoprotectant, as well as the lack of a full understanding of their mechanisms in cell protection, make selection of a single cryoprotectant difficult for different species. However, the optimal cryoprotectant can be determined empirically. The concentration usually varies in the range between 5 and 12% (v/v). Better cell protection can be achieved on employing higher concentrations, but this has to be balanced with toxicity effects of the cryoprotectant. Addition of nonpenetrating agents, such as sucrose, is generally considered to be beneficial; however, direct mixing of fish sperm with cryoprotectants inevitably leads to the death of all cells ***(33)***. Clearly, extenders play an important role in sperm protection. It has also been demonstrated for salmon sperm that multicomponent salt media are much more effective for cryopreservation ***(16)***. For marine species, the first successful protocol described by Blaxter ***(3)***, utilized a mixture of seawater and distilled water with 12.5% (v/v) glycerol as a cryoprotectant. The authors used the carp medium detailed in **Table 1** with 8% (v/v) DMSO (final concentration) for freezing marine species.
13. The level of dilution of the cryoprotectant medium is equally important and it is species sensitive ***(16)***. High-density semen needs a greater level of dilution. It was found for sturgeon sperm that the optimal ratio was 1:1 sperm:medium (0.1 *M* Tris-HCl buffer, 10% [v/v] egg yolk, 25% [v/v] DMSO] ***(17)***; however, the ratio can vary between species and even individuals. It has also been demonstrated that the concentration of sperm cells in beluga (*Huso huso*) semen may vary between males in the range of 0.58 to 6.4 billion of cells/mL and in stellate (*Acipenser stellatus*) from 0.9 to 10.4 billion of cells/mL ***(2)***. Studies on the effect of cell concentration showed that increasing carp sperm concentration from 0.5–0.8 billion/mL to 5–8 billion/mL, or 11–16 billion/mL in freezing medium resulted in a significant reduction of the temperature of ice nucleation from –4.73 ± 0.23°C to –5.75 ± 0.56°C, and –7.16 ± 2.04°C, respectively ***(34)***. We believe that a standard dilution level cannot be applied for such a broad range of sperm concentrations. For salmonids, sturgeon, carp and tilapia, milt is generally diluted between 1- and 20-fold.
14. The osmotic potential of media is inevitably substantially increased by the addition of cryoprotectant. One option that may be employed to avoid dramatic osmotic shock is the addition of egg yolk, which we have found to improve sperm survival ***(17)***. An additional factor that may be manipulated to minimize osmotic shock is the way dilutions are performed. We have observed that rapid mixing of sperm with cryoprotectant medium can damage substantive amount of cells or affect their motility. For that reason slow addition of cryoprotectant is essential for obtaining optimal results. The best sperm survival is obtained when cooled cryoprotectant medium is slowly added to sperm of equal temperature by pouring it to the wall of a slowly swirled container.

15. Equilibration time period is another important factor. Spermatozoa of salmon are relatively small and readily permeable to cryoprotectants ***(16)***. Therefore, the exposure time of sperm to cryoprotectants can be short to avoid any potential toxic effect. However, this is not the case for all species. When the motility level of freeze–thawed sturgeon sperm was compared following different equilibrating durations, it was observed that the samples that had a 40-min exposure to the cryoprotectant medium at 5°C had a significantly higher motility rate than the ones that were frozen almost immediately after placing them in cryoprotectants ***(12)***.
16. During freezing the volume of the sperm suspension expands and may consequently increase the pressure in the cryovials, potentially causing cryodamage. The increased pressure can also disrupt the hermetically sealed ampoules and thus lead to contamination of the samples. Therefore, it is important not to completely fill the vials leaving enough space for sample expansion.
17. In order to minimize the fluctuations in cooling rate when freezing in liquid nitrogen vapor, it is important to make sure that conditions of freezing are controlled appropriately. To estimate the optimal height above liquid nitrogen, where the samples are going to be held, several sperm samples should be placed at different levels above liquid nitrogen surface. The higher the sample is above the surface, the longer the freezing time should be. All samples should then be moved to approx 1 cm above liquid nitrogen for a further 10–15 min before plunging into liquid nitrogen. Assessment of motility rates in the thawed samples will indicate which holding height is optimal, and this should be used for routine cryopreservation. The cooling rate in vapor can be estimated using a thermocouple, one end of which is placed in the sample and the other in ice.
18. The cooling rate can be finely controlled by employing a controlled-rate freezer. However, it is not always practically possible to have it on the site of sperm collection. Fish sperm freezing is often performed under field conditions, where other methods of freezing, such as freezing in liquid nitrogen vapor or on dry ice, are more practical. The majority of modern controlled-rate freezers provide linear cooling rates and this may not always be the optimal cooling regime as some workers have found exponential cooling to be more effective ***(34)***. The closest easily achieved approximation to an exponential cooling regime can be achieved by using liquid nitrogen vapor-phase freezing or dry ice.
19. The authors have noted that storage of cryopreserved sperm in liquid nitrogen is possible for a long period of time without any detectable decline in sperm quality.
20. Studies on optimization of the thawing regime have demonstrated that the best thawing regime for 1–2 mL vials is using a 40°C water bath**.** As soon as sperm samples of sturgeon or salmon are thawed they should be immediately used for fertilization. Granules (*see* **Fig. 1**) can be thawed in a different way; granules are placed directly into eggs along with activating medium, the temperature of which should be the same as river water. In the case of carp, the thawed sperm samples could be stored on ice and the fertility rate could be improved after some additional treatment as described in **Note 21**.

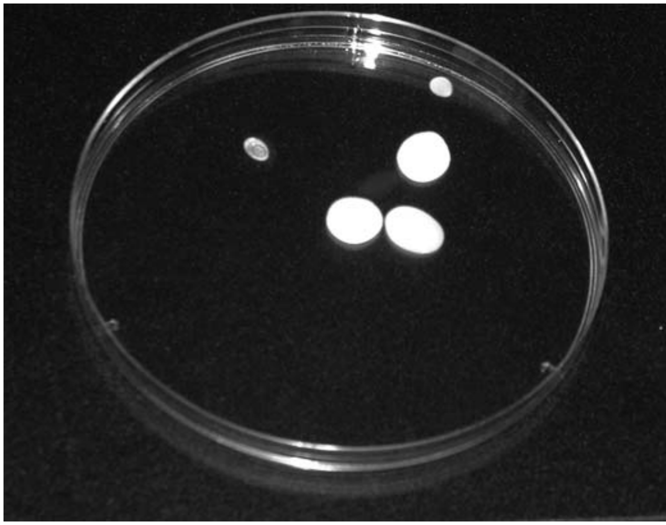

Fig.1. Sperm granules after cryopreservation.

21. Ice nucleation/crystallisation results in the removal of gases from the suspension of frozen cells. Saturation of sperm with oxygen has been reported to improve the quality of spermatozoa for cryopreserved-thawed sperm of carp ***(35)***. Significant increases in fertility were observed in all sperm samples that were aerated over 5 and 15 min at 0°C. To explain this effect the dynamics of energetic components of sperm cells was studied in fresh, sperm cryoprotectants treated and cryopreserved samples ***(36,37)***. It was found that exposure to cryoprotectants, as well as cryopreservation, affects almost all elements of the energetic system of sperm including a decrease of ATP and an increase of creatine phosphate levels ***(36,37)***.

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